THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE
Vol. 26, No. 6

Printed in U.S.A.

INTRANASAL INFECTION OF MONKEYS WITH JAPANESE ENCEPHALITIS VIRUS: CLINICAL RESPONSE AND TREATMENT WITH A NUCLEASE-RESISTANT DERIVATIVE OF POLY(I)·POLY(C)*

DONALD G. HARRINGTON, DUANE E. HILMAS, MICHAEL R. ELWELL, RICHARD E. WHITMIRE, AND EDWARD L. STEPHEN
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

Abstract. In the first experiment two rhesus (*Macaca mulatta*) and two cynomolgus (*Macaca fasicularis*) monkeys were inoculated intranasally (i.n.) with 3×10^7 plaque-forming units (PFU) of the Peking strain of Japanese encephalitis virus (JEV) to establish the time course of infection and resulting mortality. The onset of clinical signs for both species of monkeys occurred on days 5 to 9, with fever of several days duration, anorexia and depression. Death ensued in 11 to 12 days. An i.n. median lethal dose equivalent to 2.5×10^4 PFU of the Peking strain of JEV was determined in 16 additional cynomolgus monkeys. Clinical signs of infection, virus-neutralizing antibody formation, and mortality were dose-dependent for the doses of virus inoculated. Total peripheral blood leukocyte and neutrophil values increased midway during the course of infection in monkeys that died with encephalitis. Microscopic lesions of JE were similar in monkeys that died following virus challenge. No species-related differences in response to JEV challenge were evident. A nuclease-resistant complex of polyriboinosinic·polyribocytidylic acid, poly-l-lysine and carboxymethylcellulose [poly(ICLC)] reduced mortality by 50% in monkeys treated initially 8 or 24 h after virus challenge. Mean survival time of nonsurvivors was prolonged 3.5 days and microscopic lesions of encephalitis were less severe in the poly(ICLC)-treated monkeys when compared to infected-untreated monkeys. The response of rhesus and cynomolgus monkeys to JEV challenge by the i.n. route of inoculation thus provides a useful model for the study of potential antiviral compounds in host defense against Japanese encephalitis.

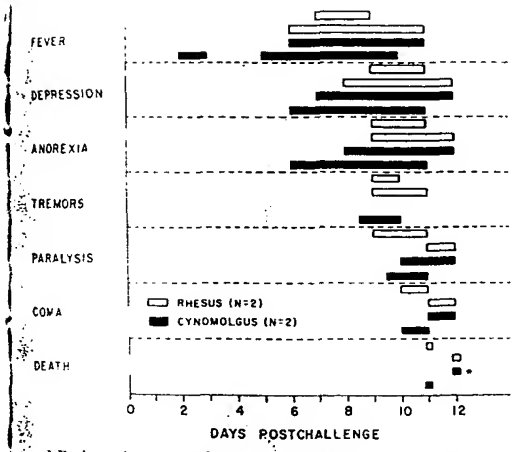
Previous studies have shown that interferon may play a role in protecting animals against Japanese encephalitis virus (JEV).[1] Mice treated with a synthetic inducer of interferon, the polyribonucleotide complex of inosinic and cytidylic acids, poly(I)·poly(C),[2] were partially protected from challenge with JEV if drug treatment was begun up to 24 h postinfection.[1] In contrast, poly(I)·poly(C) was not an effective inducer of interferon in subhuman primates, apparently because of rapid enzymatic degradation of the compound in primate serum.[3] However, a nuclease-resistant complex of poly(I)·poly(C), poly-l-lysine, and carboxymethylcellulose [poly(ICLC)] induces high levels of interferon in both monkeys and chimpanzees.[3-5] It has been used successfully to protect rhesus monkeys against both simian hemorrhagic fever[3] and yellow fever.[6] Poly(ICLC) has not been employed against any other experimental flavivirus infections which may be sensitive to interferon.

Experimental infection of monkeys and mice with JEV by the intracranial (i.c.)[7] and intranasal (i.n.)[8-10] routes of inoculation were first reported by Japanese workers several decades ago. They reported clinical signs of infection and pathologic lesions of encephalitis in monkeys similar to those described for fatal cases of JE in man.[7-14] Monkeys experimentally infected with JEV by peripheral routes other than i.n. frequently experience an immunizing infection with viremia but without clinical evidence of encephalitis.[15,16] The i.n. route of inoculation causes fatal JEV disease in monkeys without direct inoculation of virus into the central nervous system (CNS).[17,18]

The purpose of this study was to (*a*) characterize experimental JE in rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fasicularis*) monkeys

Accepted 11 April 1977.

* Address reprint requests to: MAJ Donald G. Harrington, VC, Animal Assessment Division, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, Maryland 21701.



* Euthanasia was performed on monkey when moribund.

FIGURE 1. Onset, duration, and frequency of clinical signs in two rhesus and two cynomolgus monkeys inoculated intranasally with 3×10^7 plaque-forming units of the Peking strain of Japanese encephalitis virus.

(1.5×10^6 PFU). Subsequently, the monkeys were allocated equally into three groups: poly(ICLC) treatment initiated either 8 or 24 h following virus challenge, and JEV-inoculated, untreated controls. All drug-treated monkeys received 0.3 mg/kg of poly(ICLC) intravenously once each day beginning either 8 or 24 h postchallenge and on days 5 to 9, whereas on days 2 to 4, the dosage was 3.0 mg/kg. Infected-untreated control monkeys received sham treatment once daily with an equivalent volume of pyrogen-free saline. Sera collected prior to virus inoculation and at periodic intervals after infection were assayed for JEV SN antibody.

Hematology. Blood samples for white blood cell (WBC) counts were diluted with the Unopette® System: Becton-Dickinson and Co., Rutherford, NJ) and leukocytes were counted in a hemocytometer. Differential counts were made from Wright's-stained blood smears.

Histopathology. Necropsy was performed on monkeys shortly after death; tissues from all organ systems were fixed in 10% buffered neutral formalin embedded in paraffin, sectioned at 4–6 μ and stained with hematoxylin and eosin. Neural tissues were sectioned at the lumbar and cervical spinal cord, medulla, cerebellum, cerebral cortex, thalamus, and olfactory lobes for examination.

Calculations. The i.n. LD_{50} was calculated by probit analysis[21] using survival data from study 2. Student's *t*-test was used for intergroup comparisons of mean rectal temperature and hematologic data. Significant differences are noted when $P < 0.05$.

RESULTS

Study 1: Response of rhesus and cynomolgus monkeys challenged with 3×10^7 PFU of JE (Peking) virus

Time of onset, frequency and duration of clinical signs, and time-to-death for these monkeys following i.n. virus challenge are shown in Figure 1. Clinical signs of infection appeared in the same general sequence in each monkey: fever (>39.5°C), depression, anorexia, tremors, paralysis, coma, and death (Fig. 1). The time of onset and duration of clinical signs were similar for both species. One cynomolgus monkey experienced a brief febrile response on day 2 whereas all other monkeys were febrile between days 5 and 7. The mean peak febrile response occurred on day 7 (40.4°C) in rhesus and on day 8 (40.2°C) in cynomolgus monkeys. Depression and anorexia, mild at onset and associated with fever, progressed in severity until death. The duration of febrile response was similar for both species. Rectal temperature dropped precipitously (<34°C) in all monkeys beginning 24 to 48 h prior to death. Three of the four monkeys developed slight to moderate muscular tremors of the head, trunk, and extremities by day 9. Ataxia and partial spastic paralysis of the extremities were followed in turn by inability to sit or stand, complete paralysis, and coma. The rhesus monkeys died on days 11 and 12 postchallenge, whereas one cynomolgus monkey died on day 11 and euthanasia was performed on the other when moribund on day 12. One rhesus and one cynomolgus monkey had JEV SN antibody titers of 1:10 on day 10. The other two monkeys died before SN antibody was detected.

Study 2: Intranasal LD_{50} determination

Seven of 16 cynomolgus monkeys challenged i.n. with JEV developed fever and encephalitis, and died (Table 1). These included all five monkeys in the highest dose group (1×10^5 to 10^6 PFU), 2 of 6 in the intermediate group (4×10^3 to 10^4 PFU), and none of 5 in the low (4×10^1 to 10^2 PFU) virus-dose group (Table 1). The monkey i.n. LD_{50} was 2.5×10^4 PFU. Time-to-death was

inoculated by the i.n. route with JE (Peking) virus; and (*b*) evaluate the potential effectiveness of poly(ICLC) in the therapeutic management of JE in nonhuman primates.

MATERIALS AND METHODS

Virus. Stock virus was prepared from a 5th suckling mouse brain passage of the Peking strain of JEV.[19] A 50% stock suspension containing 8×10^8 plaque-forming units (PFU) of virus/ml was prepared in 50% normal, inactivated, fetal bovine serum infusion broth and maintained at −70°C until used. Inocula were prepared by diluting the stock virus suspension in phosphate-buffered saline containing 1% heat-inactivated rabbit serum. Assays for virus in inocula were performed by a modification of the viral plaque assay technique of Rhim.[20] Serial 10-fold dilutions of inocula were made in Hank's balanced salt solution (HBSS) containing 25 mM Hepes buffer and 5% IFBS. Two-tenths milliliter of each virus dilution was inoculated onto monolayer cultures of baby hamster kidney (BHK-21) cells grown in plastic trays (9.6 cm^2/well, of Limbro Scientific Co. Inc., New Haven, Conn.). After 1.5 h incubation at 36°C in a humidified atmosphere containing 5% CO_2, samples were overlaid with 3 ml of maintenance medium[20] in 1% agarose. Four days later an additional 1.5 ml of overlay medium containing 1:5,000 w/v of neutral red was added. Plaques were counted 24 h after the second overlay. All assays were done in triplicate.

Neutralizing antibody assay. JEV serum neutralizing (SN) antibody was assayed on BHK-21 cells grown in 6-well plastic trays (9.6 cm^2/well). A mixture of 0.5 ml of virus containing approximately 100 PFU/0.1 ml and 0.5 ml of various serum dilutions was incubated at 36°C for 1.5 h, then 0.2 ml of the mixture was inoculated per well. After 1.5 h incubation at 36°C the double agar overlay was added as described above. Plaques were counted on the 5th day of incubation with 80% plaque-reduction selected as the end-point. All assays were done in triplicate.

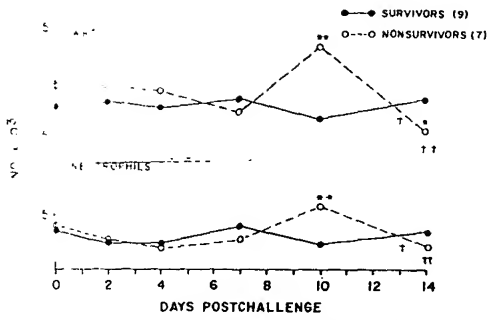
Poly ICLC. Poly(ICLC) was prepared at the National Institutes of Health as previously described.[1] The final solution contained 2 mg of poly I · poly C and bound as the complex with poly-l-lysine and carboxymethylcellulose. The complex was stored at 4°C and diluted in an equal volume of pyrogen free saline prior to use.

*Monkeys.** Healthy, well-conditioned, young adult monkeys of both sexes, (8 rhesus and 8 cynomolgus) weighing 3.6 to 4.9 and 1.9 to 3.9 kg, respectively, were used. All monkeys were negative (<1:5) for JEV serum neutralizing antibody and negative (<1:10) for JE, St. Louis encephalitis, Murray Valley encephalitis, West Nile, yellow fever and dengue serotypes 1, 2, and 3 hemagglutination inhibition antibodies prior to use. Monkeys were housed in individual cages in rooms maintained at constant temperature (25°C) with a 12-h light cycle. They were fed twice daily with commercial monkey chow (Wayne Monkey Diet, Allied Mills, Inc., Chicago, Ill.) and were provided water ad libitum. Monkeys were injected intramuscularly with 10 mg/kg of Ketaset® (ketamine hydrochloride, Bristol Laboratories, Syracuse, N.Y.) for restraint before i.n. inoculation with 0.5 ml of JEV. Half (0.25 ml) of the inoculum was given drop-wise into each nostril.

Experimental design. Three studies were conducted in which monkeys were inoculated i.n. with the Peking strain of JEV. First, two rhesus and two cynomolgus monkeys were inoculated i.n. with 3×10^7 PFU of JEV to assess their response to i.n. challenge. In the second study, 16 cynomolgus monkeys were divided at random into 4 challenge groups to determine both the response to i.n. JEV challenge and the LD_{50}. Groups were inoculated with 40 to 4×10^6 PFU of Peking strain of JEV in serial 10-fold decrements of stock virus suspension. In both studies, the experimental infection was allowed to follow its natural course. Clinical signs, including rectal temperature, depression, anorexia, tremors, paralysis, coma, were recorded daily. Femoral blood samples (approximately 3 ml) for hematologic and serologic studies were collected before and on the 3rd or 4th day following virus challenge.

The third experiment was a preliminary study to evaluate treatment with poly ICLC given i.m. in inoculation with an established uniformly lethal dose of JEV. Six rhesus monkeys were inoculated i.n. with 3 ml of JEV and

* In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as prepared by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.



† Indicates dead monkey; * $P < 0.05$; ** $P < 0.025$.

FIGURE 2. Mean total leukocyte and neutrophil values for nine surviving and seven nonsurviving monkeys inoculated intranasally with serial 10-fold dilutions of the Peking strain of Japanese encephalitis virus. The base-line values represent the mean for survivors and nonsurvivors 2 days prior to virus inoculation.

dose-related in 6 of 7 monkeys that developed clinical signs and died following virus challenge. Clinical signs of JE in these monkeys were similar to those shown in Figure 1. None of the five monkeys in the low virus-dose group were febrile or showed any clinical signs of encephalitis following virus challenge. Mean rectal temperatures for surviving and nonsurviving monkeys were significantly different ($P < 0.05$) on days 8 to 10 and 12 to 16 postchallenge. Mean peak febrile response in the nonsurviving monkeys occurred on day 9 (39.8°C), followed on day 12 by a decline in mean rectal temperature late in the course of the infection.

Mean total WBC and absolute neutrophil values were increased significantly ($P < 0.05$) on day 10 in the nonsurviving group (Fig. 2). Subsequent WBC values were decreased in four remaining monkeys on day 14 in the nonsurviving group (Fig. 2). Mean absolute lymphocyte values were significantly ($P < 0.05$) decreased on day 14 in the nonsurviving group compared with the surviving group. Three of seven nonsurviving monkeys in the high and intermediate virus-dose groups developed overt signs of infection and died before SN antibody to JEV was detected. One monkey in the intermediate virus-dose group given 4×10^3 PFU of virus showed no apparent clinical signs of illness; however, a JEV SN antibody titer of 1:10 was present on day 14. None of the five monkeys in the low virus-dose group had detectable JEV SN antibody.

Gross and microscopic lesions were similar in rhesus and cynomolgus monkeys which died during studies 1 and 2. On gross examination, all major organ systems appeared essentially normal. Significant microscopic changes of varying severity were observed within sections of brain and spinal cord of each monkey. A meningeal lymphocytic infiltrate was present over all areas of the brain and spinal cord with the most prominent foci being in the cerebellar sulci (Fig. 3). Lymphocytic perivasculitis was commonly found in both the grey and white matter of the brain and spinal cord (Fig. 4). Glial cell proliferation was pronounced in grey matter of the spinal cord, brain stem, and thalamus. Neuronal degeneration with necrosis and neuronophagia (Fig. 5) were not common in spinal cord and medulla, but were also present (although less severe) in all CNS sections. Olfactory lobes were normal except for minimal meningeal lymphocytic infiltrates and occasional

TABLE 1

Clinical findings and serum neutralizing (SN) antibody responses in cynomolgus monkeys receiving intranasal challenge with the Peking strain of Japanese encephalitis virus (JEV)

Virus-dose group	Virus-dose (PFU)	Fever* (no./total)	No. dead/total	Day of death	Reciprocal JEV antibody titer (day 14) ‡
High	4×10^6	2/2	2/2	14, 15	20, 10§
	4×10^5	3/3	3/3	15, 15, 17†	20, 5, <5
Intermediate	4×10^4	1/3	1/3	17†	<5, <5
	4×10^3	1/3	1/3	17†	<5, <5, 10
Low	4×10^2	0/3	0/3		<5, <5, <5
	4×10^1	0/2	0/2		<5, <5

* Rectal temperature ≥39.5°C.
† Monkey died with encephalitis before JEV SN antibody was detected.
‡ JEV serum neutralizing antibody inhibiting 80% plaque formation.
§ Day 14 JEV SN antibody titer.

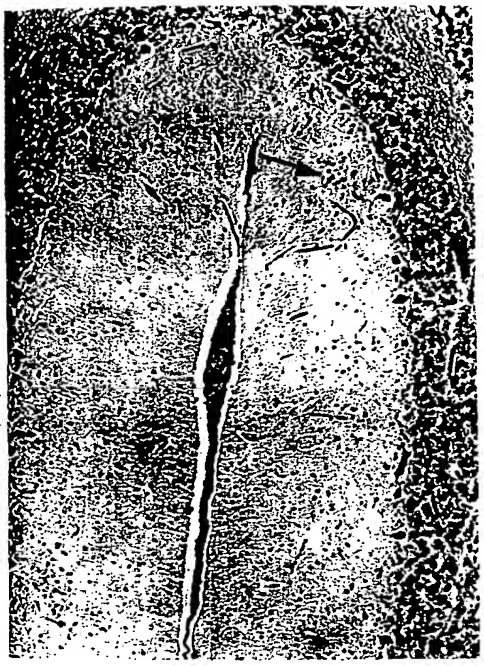


FIGURE 3. Mild lymphocytic infiltration of the meninges in a cerebellar sulcus (center) and small focus of gliosis in the molecular layer (arrow) in a monkey inoculated intranasally with the Peking strain of Japanese encephalitis virus. H & E. × 46.

perivascular cuffing. Microscopic lesions were found outside the CNS in some infected animals. Subacute myocarditis was present in two monkeys, one of which had myocardial fiber degeneration. Hemorrhage at the adrenal corticomedullary junction was seen in one monkey and lymphoid follicular degeneration was present in the spleen of several monkeys challenged with JEV.

Study 3: Effect of poly(ICLC) on JEV infection in monkeys

Two of four monkeys treated with poly(ICLC) following i.n. virus inoculation survived a lethal challenge dose of JEV. Deaths occurred in two poly(ICLC)-treated monkeys on days 18 and 19 postchallenge, one from each of the 8- and 24-h treatment groups (Table 2). Both infected-untreated monkeys died 2 to 5 days earlier than the poly(ICLC)-treated monkeys with predicted courses of infection for the challenge dose of JEV employed (Table 2). All four monkeys that died had clinical courses of infection and histopathologic lesions as described in study 2. However, lesions within the CNS and spleen of poly(ICLC)-treated monkeys were fewer and less severe when compared to lesions in the infected-untreated monkeys.

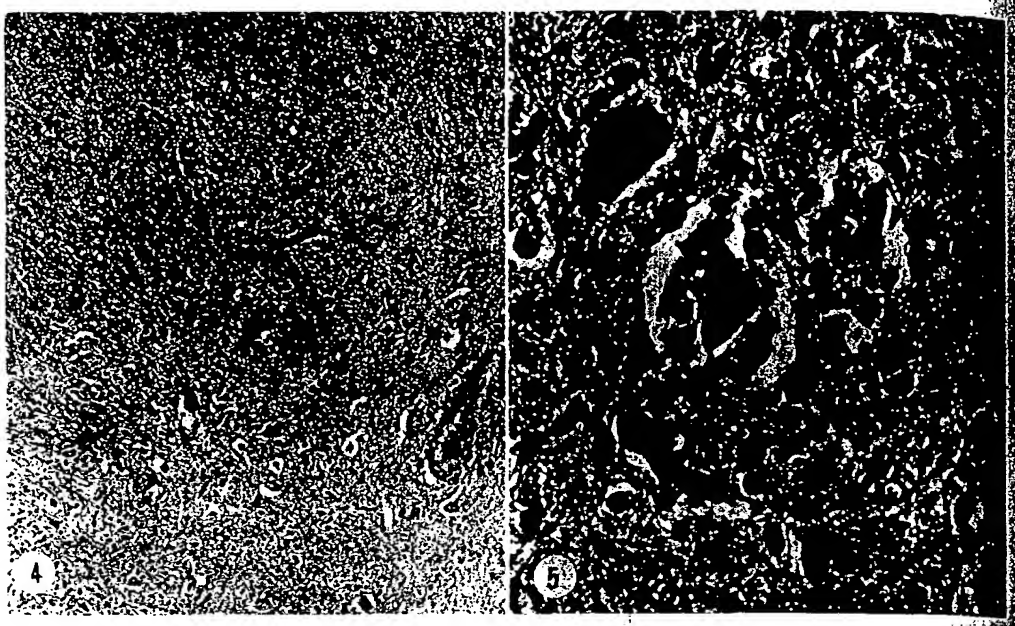
In the poly(ICLC)-treated groups, monkey A had a JEV SN antibody titer of 1:10 on day 18, the day of death, while monkey B was negative throughout the experimental period (Table 2). Monkey C, which died, and monkey D, which survived infection, had JEV SN antibody titers of 1:5 and 1:20, respectively, on day 14 (Table 2). Infected-untreated monkeys E and F died before JEV SN antibody was detectable (Table 2).

DISCUSSION

A subhuman primate model was characterized for testing the efficacy of potentially important antiviral compounds for the control of JE in man. The intranasal route of challenge was selected to induce encephalitis without inoculating the virus directly into the CNS. Clinical signs, hemograms, and histopathologic lesions are in agreement with previous reports of natural infections in man.[12,22] and experimental JEV infection in rhesus[10] and Taiwan macaques[14,15] infected by the i.n. route. Mortality in both rhesus and cynomolgus monkeys was virus-dose dependent and death occurred between 11 and 18 days postchallenge, which is consistent with previous reports of i.n. JEV challenge in macaques.[15] Approximately 4 × 10^5 PFU or more of the Peking strain of JEV was uniformly fatal in nonimmune monkeys.

Glial cell proliferation, neuronal necrosis and neuronophagia, and perivascular lymphocytic infiltration in the leptomeninges, brain, and spinal cord were lesions consistent with those reported from fatal cases of JE in monkeys following [illegible] or i.n.[8,10] challenge and in natural infections of man.[12,23] Studies in mice[8,9] and monkeys[8,10] following i.n. inoculation of JEV indicate that virus enters the brain by way of the nasal mucosa, first localizing in the olfactory lobes where it multiplies and then invades other areas of the brain and circulatory system.

Poly (ICLC) treatment for 9 consecutive days protected 2 of the 4 rhesus monkeys against a lethal challenge dose of JEV and prolonged the time-to-death of nonsurviving monkeys. Prolonged survival time and reduced severity of



FIGURES 4 and 5. Ventral horn of the spinal cord from a monkey following intranasal inoculation of Japanese encephalitis virus (Peking strain). **4.** Perivascular lymphocytic cuffing (top center) and focal gliosis (arrows). H & E, × 50. **5.** Satellitosis, neuronal necrosis, and neuronophagia (arrows). H & E, × 320.

encephalitic lesions in poly(ICLC)-treated monkeys which died indicate a partial reduction in the severity of JE infection by poly(ICLC), even when treatment was initiated 24 h following virus inoculation. To our knowledge, this is the first report which indicates that poly(ICLC), a potent interferon inducer, is beneficial in the early treatment of an otherwise fatal JEV infection in subhuman primates. It is possible that an alternative regimen of therapy might increase the protective efficacy of poly(ICLC) against JEV infection in monkeys. Protection in poly(ICLC)-treated monkeys cannot be explained by earlier and greater SN antibody than in the infected-untreated controls. Poly(ICLC) did not appear to eliminate infection in all monkeys surviving JEV challenge, as evidenced by SN antibody formation. This is in consonance with previous reports in which

TABLE 2

Effect of polyribonucleic-polyribocytidylic acid poly-L-lysine in rhesus monkeys inoculated intranasally with the Peking strain of Japanese encephalitis virus (JEV).

Time of initial treatment*	Monkey no.	Reciprocal JEV SN antibody titer† by days			Days paralyzed	Day of death
		14	18	28		
+8 h	A	<5	10	D‡	5	18
	B	<5	<5	<5	0	Survived
+24 h	C	5	20	D	5	19
	D	20	20	20	0	Survived
Untreated	E	<5	D		3	16
	F	D			2	14

* Treated monkeys received 0.3 mg/kg of poly(ICLC) intravenously once daily at 8 or 24 h post-challenge and on days 5 through 9, whereas on days 2 through 4 the dosage was 3.0 mg/g.
† JEV serum neutralizing (SN) antibody titer inhibiting 80% plaque formation.
‡ Monkey dead, serology not performed.

poly(I)·poly(C) given to mice up to 24 h following JEV inoculation increased protection against fatal encephalitis, but did not eliminate infection in all surviving animals.[1]

Control of an otherwise lethal JEV infection in two monkeys was reported following treatment with a known potent interferon inducer, poly (ICLC).[3-6] The protection afforded monkeys by poly(ICLC) is presumed to have resulted from interferon induction, although this was not determined in the study. Peking[2] and other strains[23] of JEV have been shown to be sensitive to interferon. The successful use of poly(ICLC) in the treatment of simian hemorrhagic fever[3] and yellow fever[6] infections in nonhuman primates are in general agreement with our findings for JEV infections in monkeys. The i.n. challenge model should prove useful for testing other potential antiviral compounds against JEV infection in subhuman primates. Further studies are planned to investigate different regimens of poly(ICLC) therapy.

ACKNOWLEDGMENTS

The authors thank John W. Springer, Leland E. Bolt, Rhonda D. Lambert, and Ray J. Clift for their technical assistance and Regina E. Staley for secretarial assistance. Poly(ICLC) was kindly supplied by Dr. Hilton B. Levy, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

REFERENCES

1. Postic, B., and Sather, G. E., 1970. Effect of poly I:C in mice injected with Japanese B encephalitis virus. *Ann. N.Y. Acad. Sci., 173:* 606–613.
2. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. Natl Acad. Sci., 58:* 1004–1010.
3. Levy, H. B., London, W., Fuccillo, D. A., Baron, S., and Rice, J., 1976. Prophylactic control of simian hemorrhagic fever in monkeys by an interferon inducer, polyriboinosinic-polyribocytidylic acid-poly-L-lysine. *J. Infect. Dis., 133 (Suppl.):* A256–A259.
4. Levy, H. B., Baer, G., Baron, S., Buckler, C. E., Gibbs, C. J., Iadarola, M. J., London, W. T., and Rice, J., 1975. A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. *J. Infect. Dis., 132:* 434–439.
5. Sammons, M. L., Stephen, E. L., Levy, H. B., Baron, S., and Hilmas, D. E., 1977. Interferon induction in cynomolgus and rhesus monkeys after repeated doses of a modified polyriboinosinic-polyribocytidylic acid complex. *Antimicrob. Agents Chemother., 11:* 80–83.
6. Stephen, E. L., Sammons, M. L., Pannier, W. L., Baron, S., Spertzel, R. O., and Levy, H. B., 1977. Effect of a nuclease-resistant derivative of polyriboinosinic-polyribocytidylic acid complex on yellow fever in rhesus monkeys (*Macaca mulatta*). *J. Infect. Dis., 136:* 122–126.
7. Hayashi, M., 1935. Übertragung des Virus von Encephalitis epidemica japonica auf Affen. *Folia Psychiat. Neurol. Jap., 1:* 419–465.
8. Kawamura, R., Kosama, M., Ito, T., Yazaki, T., and Kobayakawa, Y., 1936. Über das Verhalten una die Ausbreitung des encephaiitischen Virus im Zentralnervensystem bei nasal angesteckten Tieren. (Studien über den Erreger der Encephalitis epidemica. IV.te. Mitteilung.) *Trans. Soc. Pathol. Jap., 26:* 419–422.
9. Inada, R., 1937. Du mode d'infection dans l'encéphalite épidémique. *Presse Méd., 45:* 386–387.
10. Miyake, M., and Takaki, F., 1939. Morphologische Studien über die experimentelle epidemische Encephalitis (Sommerencephalitis) bei Affen (I. Mitteilung.) Über die anatomisch-histologischen Veranderungen bei nasal angesteckten Affen, insbesondere der inneren Organe. *Trans. Soc. Pathol. Jap., 29:* 110–117.
11. Takaki, F. K., 1940. Morphologische Studien über die experimentelle epidemische Encephalitis (Sommerencephalitis). (II. Mitteilung.) Über die veranderungen bei intracranial, nasal, intravenous u. subcutan angesteckten Mäusen mit besonderer Berucksichtigung der Beziehung zwischen dem Infektionsmodus und der Verteilung derselben. *Trans. Soc. Pathol. Jap., 30:* 571–575.
12. Miyake, M., 1964. The pathology of Japanese encephalitis. A review. *Bull. W.H.O., 30:* 153–160.
13. Gastaut, H., Toga, M., Bert, P., Payan, H., and Netter, J., 1960. A clinical, electroencephalographic and anatomical study of experimental Japanese "B" encephalitis in the monkey. *J. Nerv. Ment. Dis., 130:* 160–175.
14. Hsieh, W. C., Wang, S. P., and Rasmussen, A. F., Jr., 1961. Experimental infection with Japanese encephalitis virus in Taiwan monkeys. *J. Formosan Med. Assoc., 60:* 1118–1119.
15. Morris, J. A., O'Connor, J. R., and Smadel, J. E., 1955. Infection and immunity patterns in monkeys injected with viruses of Russian spring-summer and Japanese encephalitis. *Am. J. Hyg., 62:* 327–341.
16. Clarke, D. H., and Casals, J., 1965. Arboviruses; Group B. Page 627 *in* F. L. Horsfall and I. Tamm, eds., *Viral and Rickettsial Infections of Man, 4th Edition.* J. B. Lippincott Co., Philadelphia.
17. Lee, G. C.-Y., Grayston, J. T., and Wang, S.-P., 1967. Protective studies in mice and monkeys

with an inactivated Japanese encephalitis vaccine grown in hamster diploid cell culture. *Proc. Soc. Exp. Biol. Med., 125:* 803–808.

18. Lee, G. C.-Y., Grayston, J. T., and Wang, S.-P., 1971. Monkey intranasal challenge against Japanese encephalitis vaccine. Pages 87–91 *in* W. McD. Hammon, M. Kitaoka, and W. G. Downs, eds., *Immunization for Japanese Encephalitis.* Williams and Wilkins Co., Baltimore.
19. Chang, N.-C., and Liu, F.-T.,1951. Studies of epidemic encephalitis. I. Recovery and rapid identification of encephalitis virus. *Chinese Med. J., 69:* 355–360.
20. Rhim, J. S., 1962. Plaque assay of Japanese B encephalitis virus on hamster kidney monolayers. *Proc. Soc. Exp. Biol. Med., 109:* 887–889.
21. Finney, D. J., 1971. *Probit Analysis,* 3rd Edition. Cambridge University Press, Cambridge.
22. Graščenkov, N. I., 1964. Japanese encephalitis in the USSR. *Bull. W.H.O., 30:* 161–172.
23. French, G. R., and Hammond, W. McD., 1969. Studies on Japanese B encephalitis virus vaccines from tissue culture. X. Characterization of a 37°C passage line attenuated strain of OCT-541 virus. *J. Immunol., 103:* 1260–1267.

with an inactivated Japanese encephalitis vaccine grown in hamster diploid cell culture. *Proc. Soc. Exp. Biol. Med., 125:* 803–808.

18. Lee, G. C.-Y., Grayston, J. T., and Wang, S.-P., 1971. Monkey intranasal challenge against Japanese encephalitis vaccine. Pages 87–91 *in* W. McD. Hammon, M. Kitaoka, and W. G. Downs, eds., *Immunization for Japanese Encephalitis.* Williams and Wilkins Co., Baltimore.
19. Chang, N.-C., and Liu, F.-T.,1951. Studies of epidemic encephalitis. I. Recovery and rapid identification of encephalitis virus. *Chinese Med. J., 69:* 355–360.
20. Rhim, J. S., 1962. Plaque assay of Japanese B encephalitis virus on hamster kidney monolayers. *Proc. Soc. Exp. Biol. Med., 109:* 887–889.
21. Finney, D. J., 1971. *Probit Analysis,* 3rd Edition. Cambridge University Press, Cambridge.
22. Graščenkov, N. I., 1964. Japanese encephalitis in the USSR. *Bull. W.H.O., 30:* 161–172.
23. French, G. R., and Hammond, W. McD., 1969. Studies on Japanese B encephalitis virus vaccines from tissue culture. X. Characterization of a 37°C passage line attenuated strain of OCT-541 virus. *J. Immunol., 103:* 1260–1267.